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Could fecal phenylacetic and phenylpropionic acids be used as indicators of health status?

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ABSTRACT

Although most of the health effects attributed to polyphenols may be linked to their phenolic-derived metabolites, the role of the intestinal derived-phenolics in human health is still far from being well understood. We determined the profile of fecal phenolic-derived metabolites, microbiota, biomarkers of oxidative stress and inflammation, and daily intake of bioactive compounds in 71 elderly volunteers. Phenylacetic and phenylpropionic acids were the main phenolic metabolites present in feces. From them, phenylacetic acid was related with a more pro-oxidant and immune stimulated status, and both were negatively associated with fecal propionate, whereas phenylpropionic acid was directly related with the fecal concentration of acetate. Moreover, phenylacetic acid was negatively associated with the *Bacteroides* group and *Clostridium* cluster XIVa and positively with *Lactobacillus*. These results provide a rationale to explore the potential of fecal microbial phenolic-derived metabolites as possible biomarkers of health status in future studies focused on the elderly population.

KEYWORDS

Polyphenols; fecal phenolic derived-metabolites; gut microbiota; short chain fatty acids; biomarkers.

INTRODUCTION

Dietary (poly)phenols are bioactive compounds of vegetal origin that have been receiving considerable deal of attention from the scientific community in the last years. Most of the mechanisms proposed for their putative protective effects against the development of several chronic conditions such as cardiovascular diseases[1–4] or cancers[5–8] are based on their role as scavengers of free radicals but also on their capability to reduce host cellular proliferation and to act as anti-inflammatory agents [9–11]. Polyphenols present in foods are poorly absorbed in the small intestine and a substantial proportion of them reach the colon after digestion where, by de-esterification, hydrogenation, demethylation and/or dehydroxylation, they are transformed by the microbiota into different derived metabolites of low molecular weight,[12–14] which are often better absorbed than the parent compounds.

There is an increasing body of evidence suggesting that a significant part of the health effects attributed to fruits, vegetables or drinks such as red wine, coffee or tea may be linked to their polyphenol content through their phenolic-derived intestinal microbial metabolites, not occurring preformed in the diet.[15] In this regard, changes in the phenolic profile of human feces have been reported after the intake of polyphenol-rich foods such as red wine,[13,16] pomegranate juice,[17] raspberry,[18] or following supplementation with isoflavones.[19] The results obtained so far evidenced a considerable inter- and intra-individual variation in the biological response to polyphenols, that could be attributed to the different dietary patterns and to the existing diversity in the colonic microbiota of the different subjects [13,16,19,20].

However, while it has been reported that dietary polyphenols can inhibit certain intestinal pathogenic microorganisms[16,21] and/or stimulate the proliferation of specific beneficial microbes,[21] thus contributing to the maintenance of a healthy microbial balance in the gut,[14] there is still scarce information in the literature regarding the interrelationship between the intestinal phenolic compounds and the whole intestinal microbiota. Some

previous *in vitro* studies have shown that the concentrations of benzoic acid, phenylacetic acid, phenylpropionic acid and 3-(3'-hydroxyphenyl)-propionic acid quantified in human fecal water were present at levels large enough to influence intestinal bacterial growth.[22] Then, the characterization of the fecal microbial-derived phenolic metabolites is of interest for a better understanding of the metabolism of phenolic compounds by gut bacteria and its consequences for human health.

Our aim in the present work was to examine the fecal phenolic profile in the feces of a sample of mature subjects without a declared pathology, and to determine their possible associations with fecal microbiota. We have also evaluated whether the excretion of phenolic catabolites was influenced by fibers and dietary phenolic compounds as well as their possible association with serum parameters related with oxidative stress, inflammation and immune status. This global and multidisciplinary approach could be of help for advancing in the knowledge about the effect of polyphenols on human health, by means of generating new hypotheses that could be tested in future studies.

MATERIALS AND METHODS

Participants

The sample of the study includes seventy-one healthy, mature volunteers (51 women and 20 men; 70.83 ± 11.12 years old) recruited between 2010 and 2012 in the Asturias region (North of Spain), without previous diagnosis of cancer, autoimmune or gastrointestinal diseases, and neither consumption of antibiotics or probiotics/prebiotics one month prior to the study. All subjects were mentally and physically capable to participate in the study and gave informed written consent. Ethical approval was obtained from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias, Ref. no. 17/2010), in compliance with the Declaration of Helsinki.

Nutritional assessment

Dietary intake has been registered by a personal interview using an annual, semi quantitative Food Frequency Questionnaire (FFQ) which has been designed *ad hoc* for the purpose of this study and validated for dietary fibers and polyphenols by means of a 24 h recall method. During a personalized interview, volunteers were asked, by expert dieticians, item by item, whether they usually ate each food and, if so, how much they ate. Methodological issues concerning dietary assessment have been described previously.[23] Food intake was analyzed for energy, macronutrients, and total dietary fiber content by using the nutrient Food Composition Tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID).[24] Also, the following fiber components were ascertained using the Marlett et al. food composition tables[25]: soluble fiber, soluble pectin, soluble hemicellulose, insoluble fiber, insoluble pectin, insoluble hemicellulose, Klason lignin, and cellulose, based on the enzymatic-chemical method developed by Theander et al. by which pectin content is determined using calorimetric assay, cellulose and hemicellulose are determined by high-performance liquid chromatography (HPLC),[26] and Klason lignin is estimated as the insoluble material after a

Saeman acid hydrolysis.[27] The polyphenols content in foods was completed using the Phenol Explorer database that contains detailed information from over 400 foods consumed regularly in European countries[28] and data about the oxygen radical absorbance capacity (ORAC) of foods was obtained from the database from the ORAC of select foods from USDA.[29] During the personal interview, information was also collected on potential confounders such as smoking habits ("Do you smoke?"), alcohol intake ("How much alcohol do you consume during the day?") or physical activity ("How many time do you spent daily on physical activity?), previously associated with phenolic excretion,[30] and regarding bowel habits by registering the number of depositions per week and the consistence of feces.

Height of the participants was measured using a stadiometer with an accuracy of ±1 mm (Año-Sayol, Barcelona, Spain). The subjects stood barefoot, in an upright position and with the head positioned in the Frankfort horizontal plane. Weight was measured on a scale with an accuracy of ±100 g (Seca, Hamburg, Germany). Body mass index (BMI) was calculated using the formula: weight (Kg) / height $(m)^2$.

Blood biochemical analyses

An overnight fast blood sample was drawn by venepuncture after a 12-hour fast and collected in separate tubes for serum and plasma. Samples were kept on ice and centrifuged (1000 × g, 15 minutes) within 2–4 hours after collection. Plasma and serum aliquots were kept at -20 °C until analyses were performed. Serum glucose, serum total cholesterol, serum HDL-cholesterol, serum LDL-cholesterol and serum triglycerides were determined by using an automated biochemical auto-analyser.

Total antioxidant capacity (TAC) in serum was determined by the colorimetric assay P40117 (Innoprot, Innovative Technologies in Biological Systems, Vizcaya, Spain). This method determines the conversion of, Cu^{2+} to Cu^{+} by serum small molecules and proteins.

The reduced ion is chelated with a colorimetric probe, giving a broad absorbance peak around 450 nm, which is proportional to the TAC.[31] Serum malondialdehyde (MDA) concentrations were determined by the spectrophotometric method of lipid peroxidation LPO-586 (Byoxytech, Oxis International, Portland, OR).[32] Serum levels of C-reactive protein (CRP) were determined by CRP Human Instant ELISA (eBioscience, San Diego, CA). Levels of serum IL-10, IL-8, IL-17, TNF-α, and IL-12 were quantified by flow cytometry using a multiplex immunoassay (Cytometric Bead Array, CBA, BD Biosciences). The concentration of transforming growth factor (TGF-β) was determined by ELISA (BD OptEIA^{TM}, BD Biosciences).

Fecal samples collection and processing

Feces were collected in an interval of 7 days after the nutritional interviews. Fresh samples were collected, placed in a sterile container (provided to the volunteers by the research team, together with sterile tools to facilitate sample management) and immediately frozen at -20 °C (in the home freezer). Then, the samples were transferred (frozen at -80 °C) to the laboratory.[33,34] Prior to analyses fecal samples were melted, one gram of sample was weighed, diluted 1:10 in sterile phosphate-buffered saline solution (PBS) and homogenized in a Lab-Blender 400 stomacher (Seward Medical, London, UK) at full speed for 4 min. One mL of the homogenized samples was centrifuged (10,000g, 30 min, 4 °C). The pellet obtained was then used for fecal microbiota DNA extraction whereas the supernatant was filtered through 0.2 μm filters, mixed with 1/10 of ethyl butyric acid (1 mg/mL) as an internal standard and stored at −80 °C until gas chromatography (GC) analyses were performed. Sample preparation was carried out in duplicate.

Fecal microbiota analyses

Fecal DNA was obtained from by using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) as previously described.[34] PCR amplification and detection of the 16S rRNA

gene for the quantification of different bacterial groups (*Akkermansia, Bacteroides–Prevotella–Porphyromonas* group, *Bifidobacterium, Clostridium* cluster XVIa, *Lactobacillus* group and *Faecalibacterium*) was performed in a 7,500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems) as described before.[35] Samples were analysed in duplicate in two independent PCR runs.

Short fatty acids analyses

Analysis of SCFA (acetate, propionate, isobutyrate, butyrate, and isovalerate) was performed in a gas chromatograph 6890N (Agilent Technologies Inc, Palo Alto, CA, USA) connected to a mass spectrometry (MS) 5973N detector (Agilent Technologies) and to a flame ionization detector (FID) as described previously.[36]

Targeted analysis of phenolic metabolites in feces

For sample preparation, frozen fecal samples were thawed at room temperature, and one gram was taken, diluted 1/10 in sterile phosphate-buffered saline solution (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, prepared from tablets from Sigma-Aldrich), and homogenized in a LabBlender 400 stomacher (Seward Medical, London, U.K.) at full speed for 4 min. Supernatants were then obtained by centrifugation (10000g, 30 min, 4 °C) and filtration through 0.2 μm and stored at −20 °C until analysis. An internal standard 4-hydroxybenzoic-2,3,5,6-d4 acid solution (Sigma-Aldrich, St. Louis, MO) [1250 μg/mL in formic acid/acetonitrile (1:200, v/v)] was added to the samples in a proportion 1:5 (v/v). Sample preparation was carried out in duplicate.

For the analysis of phenolic metabolites in the fecal solutions, a previously reported UPLC-ESI-MS/MS method was followed,[16,37] with some modifications. The limit of detection of phenolic acids by this UPLC-TQMS equipment is up to 0.001 μg/mL.[38] The

159 liquid chromatographic system was a Waters Acquity UPLC (Milford, MA) equipped with
160 a binary pump, an autosampler thermostated at 10 °C, and a heated column compartment
161 (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm and 1.7 μm particle size,
162 from Waters (Milford, MA, USA). The mobile phases were 0.1% (v/v) formic acid in
163 water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The gradient program was as
164 follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14
165 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium
166 time was 2.4 min, resulting in a total run time of 18 min. The flow rate was set constant at
167 0.5 mL/min, and the injection volume was 2 μL. The LC effluent was pumped to an
168 Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray
169 ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as
170 follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400
171 °C; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. For
172 quantification purposes, data were collected in the multiple reaction monitoring (MRM)
173 mode, tracking the transition of parent and product ions specific to each compound. The
174 MS/MS parameters (cone voltage, collision energy, and MRM transition) of the 62
175 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols,
176 hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-
177 hydroxyvaleric acids, and valerolactones) were previously reported.[16] The ESI was
178 operated in negative ionization mode, except for γ-valerolactone (positive mode). All
179 metabolites were detected using the calibration curves of their corresponding standards,
180 commercially available from different suppliers (Sigma-Aldrich Chemical Co., St. Louis,
181 MO; Phytolab, Vestenbergsgreuth, Germany; and Extrasynthese, Genay, France), except
182 for 4-hydroxy-5-(4′-hydroxyphenyl)valeric and 4-hydroxy-5-(3′,4′-
183 dihydroxyphenyl)valeric acids, which were quantified using the calibration curves of 3-(4′-
184 hydroxyphenyl)- propionic and 3-(3′,4′-dihydroxyphenyl)propionic acids, respectively.

Data acquisition and processing was realized with MassLynx 4.1 software. Results are expressed as the amount (μg) of phenolic metabolites in 1 mL of decimal fecal dilutions. All analyses were performed in duplicate.

Statistical analyses

IBM-SPSS version 22.0 (SPSS-Inc., Chicago) was used for statistical analyses. Goodness of fit to normal distribution was analyzed with the Kolmogorov-Smirnov test. When the distribution of variables was skewed, the natural logarithm of each value was used in the statistical test. The variable total phenolic metabolite was calculated by the sum of the fecal compounds detectable in at least 35 subjects of the sample. A Student's t-test was used to evaluate the differences in continuous variables according to the tertile of total phenolic metabolite content in feces, whilst categorical variables were examined using chi-squared analysis. Also, the linear trend between these variables was explored by means of linear regression analysis adjusting for age, BMI, energy intake, and physical activity as covariates. To deepen into the associations between the diet and the excretion of major phenolic metabolites in feces, a Spearman correlation analysis was conducted. Heatmap was generated under R version 3.3.3 package heatmap.2. The conventional probability value for significance (0.05) was used in the interpretation of results.

RESULTS

The main phenolic metabolites determined by UPLC-ESI-MS/MS in feces were phenylacetic and phenylpropionic acids, accounting, on average, for 46.7 and 35.4%, respectively, of the total phenolic metabolites excreted in feces. For analyzing the data, the sample was divided according to the levels of total phenolic metabolites excreted in feces into tertiles: high (tertile 3), medium (tertile 2), and low (tertile 1) (Table 1). The general characteristics of the sample population were similar across the tertiles, with the exception of the contribution of proteins and lipids to the total energy intake, which was higher for both macronutrients in the individuals included in tertile 1 (lowest total phenolic excretory levels in Table 1). As expected from the division of the sample in tertiles, the levels of the majority phenolic compounds, i.e. phenylacetic and phenylpropionic acids, and to a lesser extent 3-(3′-hydroxyphenyl) propionic acid, displayed a clear trend to increase from tertile 1 to tertile 3, with statistically significant differences among tertiles (Table 2). However, such trend was not so clear for the minority metabolites determined (Table 2). This prompted us to focus on the majority phenolic metabolites: phenylacetic, phenylpropionic and 3-(3′-hydroxyphenyl) propionic acids.

Then, we looked for a possible association between the intestinal microbial groups and SCFA quantified, with the three-major fecal phenolic metabolites as well as with the total phenolics content excreted in feces (Table 3). Total phenolic metabolite content was inversely associated with the fecal levels of *Bacteroides* group, *Clostridium* cluster XIVa and propionate, and directly related with *Lactobacillus* group and acetate. Phenylacetic acid showed a negative association with *Bacteroides* group and *Clostridium* cluster XIVa and propionate, and was positively related with *Lactobacillus* group. An inverse association was also found between phenylpropionic acid excretion and the levels of propionate, while this phenolic metabolite showed a direct association with acetate. In

addition, 3-(3´-hydroxyphenyl) propionic acid was inversely related with isovalerate levels.

Linear regression analyses were also conducted in order to investigate the possible associations between the excretion of phenolic metabolites and some blood biomarkers (Table 4). The results obtained pointed to a direct association of TGF-β, IL-17 and IL-8 levels with the total phenolics excretion in feces, with independence of age, energy intake, physical activity and BMI. Protocatechuic and phthalic acids have shown a positive relationship with TGF-β, and phthalic acid with IL-8. The excretion of phenylacetic acid was directly related with serum biomarkers such as MDA and C-reactive protein, and with immune parameters as TGF-β, IL-10, IL-17 and IL-8.

To deepen into the possible associations between the majority fecal phenolics excretion and diet, we looked for correlations between the main fecal phenolic metabolites, and the intake of dietary compounds (Figure 1). Phenylacetic acid was inversely related with the intake of some flavonoids and phenolic acids and showed a positive association with isoflavonoids, kaempferol 3-O-glucoside, kaempferol 3-O-xylosyl-glucoside, kaempferol 3-O-acetyl-glucoside, procyanidin dimer B5 and p-coumaroylquinic acid. Whereas phenylpropionic acid was directly associated with the intake of different proanthocyanidins and soluble fiber, 3-(3′-hydroxyphenyl) propionic acid was related with insoluble fibers, flavones and flavanols. The total fecal phenolic metabolite content showed a directly correlation only with kaempferol 3-O-glucoside. Moreover, the dietary polyphenols, statistically related with phenolic excretion, were directly associated with the intake of different types of fibers (Figure 2).

DISCUSSION

Our data provide new and valuable information about the link between the major phenolic metabolites in feces and the gut microbiota composition in the context of a low-grade pro-oxidant and pro-inflammatory status of the host, as frequently occurs at advanced age.

It is not completely clear how changes in the profile and levels of fecal phenolic metabolites may be related to their biological effects. In this sense, there is a general consensus in the literature supporting the importance of the aqueous phase components of the human feces for modulating the colonic environment,[39,40] the profile and levels of phenolics being a direct indicator of the microbial phenolic degradation products.[41] However, to be effective at the physiological level it is necessary that phenolics are absorbed and reach target tissues. Nonetheless, the absorption of phenolics is difficult to predict from the excreted fraction, since they only represent the metabolites non-absorbed.[42] To date, some authors have suggested that higher total phenolic excretion in feces could be directly related with a higher concentration of these bioactive compounds at the intestinal level[43] which would imply a greater protection against oxidative stress and the action of potential carcinogens.[12] From the analytic point of view, stool is an easily accessible and non-invasive matrix with metabolites originating from host, its gut microbiota, and food components. Therefore, analysis of stool samples is a good approach to ascertain how phenolic profile and content in intestinal fluids can be influenced by the diet.[44] Since not all phenolic-derived metabolites are augmented in the feces of those individuals displaying the highest total phenolic excretory levels as compared to the excretors of lower levels, it is possible that differential effects among the distinct fecal phenolics could exist. From the evaluated metabolites, phenylacetic and phenylpropionic acids were by far the most abundant. Thus, hereinafter we will focus our discussion on these two metabolites, considered individually, and their possible differential impact on human health. In agreement with previous studies from other authors,[45,46] we identified

phenylacetic acid as the most abundant phenolic metabolite in human feces, explaining approximately the 45% of the total phenolic excretion.

Whereas other phenolic metabolites in feces are predominantly derived from the microbial metabolism of polyphenols contained in vegetable foodstuffs, phenylacetic acid is mostly derived from the intestinal microbial fermentation of aromatic amino acids, particularly phenylalanine through the phenylpropanoid pathway[47] as well as from endogenous production.[37] The association of phenylacetic acid with a more pro-oxidant and pro-inflammatory status found in the present work supports our previous results suggesting an association of this phenolic acid with variables related with an "unhealthy lifestyle" and obesity[30]. At this point, it may be interesting to consider whether the higher fecal levels of this compound could be the cause or the reflection of a pro-inflammatory status. Based on evidences provided by other authors, the last option seems plausible since the pro-inflammatory status often observed in relation with advanced age (the so-called *inflammaging*) has been shown to be associated with an altered tyrosine metabolism in elderly persons.[48] Phenylacetic acid is an endogenous intermediate catabolite of phenylalanine and therefore, variations in the levels of phenylacetic acid could be reflecting changes in the endogenous amino acids metabolism. On the other hand, increased levels of phenylalanine at the expenses of tyrosine have been associated with a chronic low-grade inflammation in elderly persons,[48] thus providing a link between phenylacetic acid and the immune status of mature adults. In this scenario, we wanted to examine whether the differences in phenylacetic acid excretion could be also related with diet and the intestinal microbiota profile. Data available in the literature concerning the phenolic metabolites produced by microbial colonic degradation are scarce. However, the association found by us between the intake of procyanidin dimer-B5 and phenylacetic acid excretion is in consonance with the direct association between the fecal levels of lactobacilli and this phenolic metabolite, and with previous data by other authors reporting

that some microorganisms from the genus *Lactobacillus* are able to transform procyanidins with a lower degree of polymerization into phenylacetic acid.[49]

On the other hand, supporting the evidences about the impact of the daily intake of polyphenols on the gut microbiota, modulating its composition and/or functionality,[50] we have found a positive association between phenylpropionic acid and the intake of different proantocyanidins and soluble fiber. Considering the high correlation found by us between most of these dietary compounds and soluble fibers (i.e.: narigenin r=0.670, apigenin r=0.538, lariciresinol r=0.713, 5-caffeoylquinic acid r=0.304, (-)-epicatechin-3-O-gallate r=0.388, $p \leq 0.01$, Figure 2), it might be possible that fibers could interact with the fecal microbial metabolism of these phenolics by increasing the levels of proanthocyanidins reaching the colon, thus favoring their metabolization by the intestinal microbiota and the formation of SCFA.[51-53] Finally, an inverse association between the concentration in feces of total phenolic metabolites, and the concentration of propionate and levels of *Bacteroides* group and the *Clostridium* cluster XIVa, was found in the present work. In this regard, reduced levels of the major butyrate producer microorganisms in the human colon (*Clostridium* cluster IV that includes *Faecalibacterium* genus, and *Clostridium* cluster XIVa) as well as of the genus *Bacteroides* has been repeatedly reported situations in which the oxidative status may be altered.[54-57] Moreover, alterations on fecal levels of *Bacteroides* and butyrate-producing bacteria as well as increased levels of *Lactobacillus* have been recently communicated by us in over-weight and obese individuals from the general population that also presented a more pro-oxidant and pro-inflammatory status.[58] In addition, an intestinal microbiota imbalance has been linked with some states associated to obesity and insulin resistance in which the relative proportion of acetate is increased,[59,60] similarly as to what occurs in the present work for the positive association found between total fecal phenolic-derived compounds and fecal acetate concentration. In contrast to what has been indicated so far, Russell et al.[47] have reported that microbial protein fermentation

is the likely source of phenylacetic acid and other phenylpropanoid-derived metabolites in the human colon, aromatic amino acid-metabolizing activity being particularly prevalent among *Bacteroides* spp. and to a considerable lesser extent among some members of the *Clostridium* cluster XIVa.[47] Therefore, the inverse association found by us between fecal phenylacetic acid and these two microbial groups and propionic acid (a metabolite mainly produced by *Bacteroides*) could also be due to variations in the metabolic activity among the microbiotas of individuals as related to the fecal phenolics profile.[61]

This study presents some strengths and limitations that deserve additional comment. The holistic approach and the use of well-validated and efficient UPLC-MS methodologies for fecal metabolite analysis are strengths of the present study. On the other hand, although FFQ has a limited capacity for accurately quantify the daily intake, it is however at present the most suitable method available to describe regular dietary habits.[62] This aspect is of great importance for the study of the relationship between diet and the microbial intestinal environment since usual diets are known to be the main drivers that shape the microbial composition and metabolic activity of the intestinal ecosystem. Giving the transversal design of the present study, a directionality in the observed associations cannot be established. Nevertheless, we have identified two main different metabolic profiles as related with the differential excretion of total fecal phenolic compounds and their association with serum biomarkers and with gut microbial composition. This opens the possibility of designing future studies in order to explore the potential of phenylacetic and phenylpropionic acids as possible biomarkers of health status and/or as markers of the individual gut microbiota composition and functionality in both health and illness states. This would help in reinforcing the promotion and maintenance of a healthy status through diet.

ABBREVIATIONS USED

BMI, body mass index; CESNID, Centro de Enseñanza Superior de Nutrición Humana y Dietética; ESI, Z-spray electrospray ionization; FID, flame ionization detector; FFQ, food frequency questionnaire; GC, gas chromatography; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; MS, mass spectrometry; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; PCR, C-reactive protein; SCFA, short chain fatty acids; TAC, total antioxidant capacity; TGF-β, transforming growth factor, UPLC-ESI-MS/MS, ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry.

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CONFLICT OF INTEREST STATEMENT

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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595 **FIGURE CAPTIONS**

Figure 1. Spearman correlation between those major fecal phenolic metabolites (μg/mL) whose concentration differed significantly among tertiles, with dietary polyphenols (mg/day) and fibers (g/day). Columns correspond to fecal microbial metabolites; rows correspond to dietary compounds. Blue and red colors denote negative and positive association, respectively. The intensity of the colors represents the degree of association between these phenolic compounds determined in feces and several dietary compounds and asterisk indicate significant associations: *$p < 0.05$; ** $p \leq 0.01$.

Figure 2. Spearman correlation between dietary polyphenols (mg/day) and the different classes and subclasses of dietary fibers (g/day). Columns correspond to the different classes of fibers; rows correspond to dietary polyphenols. Blue and red colors denote negative and positive association, respectively. The intensity of the colors represents the degree of association between variables and asterisk indicate significant associations: *$p < 0.05$; ** $p \leq 0.01$. Only significant results were presented.

TABLES

Table 1. General Characteristics of the Sample According to the Tertiles Formed Considering the Total Phenolic Metabolite Content in Feces[a].

	total phenolic metabolite content (μg/mL)		
	tertile 1[b] (n=24)	tertile 2[c] (n=23)	tertile 3[d] (n=24)
age (y)	$73.0 \pm 12.6_a$	$69.4 \pm 11.1_a$	$70.0 \pm 9.8_a$
female (% of subjects)	66.7_a	78.3_a	70.8_a
BMI[e] (kg/m^2)	$27.7 \pm 4.5_a$	$26.1 \pm 3.1_a$	$28.1 \pm 4.1_a$
sedentary lifestyle (% of subjects)	54.2_a	52.2_a	66.7_a
non-smoker (% of subjects)	70.8_a	65.2_a	83.3_a
deposition (times/week)	$5.7 \pm 2.1_a$	$6.4 \pm 2.3_a$	$6.6 \pm 2.3_a$
blood parameters			
serum glucose (mg/dL)	$98.9 \pm 18.6_a$	$98.5 \pm 17.1_a$	$105.7 \pm 32.3_a$
total cholesterol (mg/dL)	$212.5 \pm 34.5_a$	$217.6 \pm 47.5_a$	$224.6 \pm 41.8_a$
HDL-cholesterol[f] (mg/dL)	$53.1 \pm 11.1_a$	$54.1 \pm 15.7_a$	$52.8 \pm 12.4_a$
LDL-cholesterol[g] (mg/dL)	$138.5 \pm 29.4_a$	$140.4 \pm 39.4_a$	$143.7 \pm 36.5_a$
Ttriglycerides (mg/dL)	$104.2 \pm 30.4_a$	$115.3 \pm 51.1_a$	$140.6 \pm 63.2_a$
TAC[h] (mM)	$0.3 \pm 0.1_{a,b}$	$0.3 \pm 0.1_a$	$0.4 \pm 0.1_b$
MDA[i] (μM)	$2.3 \pm 0.6_a$	$2.2 \pm 0.5_a$	$2.5 \pm 0.6_a$
CRP[j] (pg/mL)	$1.2 \pm 1.3_a$	$0.9 \pm 0.8_a$	$1.6 \pm 1.2_a$
diet			
energy intake (kcal/day)	$1842.7 \pm 445.9_a$	$1790.5 \pm 425.9_a$	$1851.7 \pm 627.6_a$
carbohydrates (% of total energy)	$39.3 \pm 5.7_a$	$40.2 \pm 6.4_a$	$42.5 \pm 6.7_a$
lipids (% of total energy)	$38.9 \pm 3.9_a$	$38.6 \pm 6.8_{a,b}$	$35.6 \pm 5.8_b$
proteins (% of total energy)	$20.9 \pm 3.6_a$	$18.7 \pm 2.4_b$	$19.3 \pm 2.7_{a,b}$
polyphenols (mg/day)	$1338.1 \pm 1268.2_a$	$1690.8 \pm 1081.4_a$	$1592.2 \pm 1292.3_a$
fiber (g/day)	$19.1 \pm 7.5_a$	$18.6 \pm 6.2_a$	$21.15 \pm 10.4_a$
insoluble fiber (g/day)	$11.9 \pm 5.6_a$	$11.4 \pm 4.3_a$	$13.9 \pm 6.4_a$
ORAC[k] (μmol TE[l]/day)	$7096.7 \pm 6185.2_a$	$7387.9 \pm 6256.1_a$	$7300.4 \pm 7910.6_a$

[a]Results derived from Student's *t*-test are presented as estimated marginal mean ± standard deviation and differences in categorical variables are examined using chi-squared analysis and presented as percentage (%). Range of phenolics in feces according to tertiles: [b]tertile 1 (3.0-15.9 μg/mL), [c]tertile 2 (15.9-22.7 μg/mL) and [d]tertile 3 (22.7-109.0 μg/mL). Different subscript letters indicate significant statistical differences ($p \leq 0.05$). [e]BMI, body mass index. [f]HDL, high-density lipoprotein. [g]LDL, low-density lipoprotein. [h]TAC, total antioxidant capacity. [i]MDA, malondialdehyde. [j]CRP, C-reactive protein. [k]ORAC, oxygen radical absorbance capacity. [l]TE, Trolox equivalents.

Table 2. Concentration of the Major Fecal Phenolic Metabolites in the Population According to the Tertiles Formed Considering the Total Phenolic Excretion Levels[a].

	phenolic metabolite content (μg/mL)		
	tertile 1[b] (n=24)	tertile 2[c] (n=23)	tertile 3[d] (n=24)
benzoic acids (μg/mL)			
protocatechuic acid	$0.089 \pm 0.085_{a}$ (19)	$0.111 \pm 0.072_{a}$ (19)	$0.233 \pm 0.253_{b*}$ (24)
4-hydroxybenzoic acid	$0.267 \pm 0.380_{a}$ (21)	$0.169 \pm 0.133_{a}$ (23)	$0.172 \pm 0.144_{a}$ (24)
benzoic acid	$0.913 \pm 0.267_{a}$ (13)	$1.022 \pm 0.363_{a}$ (15)	$1.409 \pm 1.048_{a}$ (15)
phthalic acid	$0.213 \pm 0.254_{a,b}$ (11)	$0.157 \pm 0.114_{a}$ (13)	$0.560 \pm 0.876_{b*}$ (23)
phenylacetic acids (μg/mL)			
phenylacetic acid	$4.416 \pm 1.714_{a}$ (22)	$7.439 \pm 2.236_{b**}$ (22)	$25.201 \pm 22.471_{c**}$ (24)
3-hydroxyphenylacetic acid	$0.448 \pm 0.365_{a}$ (11)	$0.475 \pm 0.536_{a}$ (18)	$0.759 \pm 0.728_{a}$ (22)
4-hydroxyphenylacetic acid	$0.701 \pm 1.196_{a}$ (13)	$0.508 \pm 1.068_{a}$ (18)	$0.517 \pm 0.423_{a}$ (20)
phenylpropionic acids (μg/mL)			
3-(3′-hydroxyphenyl)propionic acid	$0.536 \pm 1.043_{a}$ (21)	$1.410 \pm 3.837_{a,b}$ (23)	$2.612 \pm 4.450_{b*}$ (24)
phenylpropionic acid	$6.936 \pm 0.627_{a}$ (10)	$8.588 \pm 1.900_{b**}$ (22)	$16.607 \pm 8.890_{c**}$ (24)
cinnamic acids (μg/mL)			
p-coumaric acid	$0.045 \pm 0.052_{a}$ (17)	$0.040 \pm 0.053_{a}$ (17)	$0.071 \pm 0.075_{a}$ (23)
ferulic acid	$0.028 \pm 0.029_{a}$ (14)	$0.021 \pm 0.017_{a}$ (14)	$0.135 \pm 0.244_{a}$ (17)

[a]Results derived from Student's *t*-test are presented as estimated marginal mean ± standard deviation. Range of phenolic metabolites in feces according to tertiles: [b]tertile 1 (3.0-15.9 μg/mL), [c]tertile 2 (15.9-22.7 μg/mL) and [d]tertile 3 (22.7-109.0 μg/mL). Different subscript letters indicate significant statistical differences. *$p \leq 0.05$; ** $p \leq 0.01$.

Table 3. Lineal Regression Analysis between the Fecal Microbial Groups and Short-Chain Fatty Acids (SCFA) and the Excretion of those Fecal Phenolic Metabolites whose Concentration Differed Significantly among Tertiles[a].

	total phenolic content (μg/mL)		phenylacetic acid (μg/mL)		3-(3′-hydroxyphenyl) propionic acid (μg/mL)		phenylpropionic acid (μg/mL)		protocatechuic acid (μg/mL)		phthalic acid (μg/mL)	
	R^2 [b]	β[c]	R^2	β	R^2	β	R^2	β	R^2	β	R^2	β
Microbiota[d] (Log No. Cells per Gram feces)												
Akkermansia	0.233	-0.079	0.257	0.047	0.204	-0.122	0.101	-0.197	0.118	-0.004	0.237	0.184
Bacteroides-Prevotella-Porphyromonas		-0.592*		-0.675*		-0.258		-0.157		-0.082		-0.650*
Bifidobacterium		0.429		0.489		0.276		0.040		0.102		0.437
Clostridium cluster XIVa		-0.475*		-0.491*		-0.176		-0.193		-0.238		-0.539*
Lactobacillus group		0.578*		0.555*		0.262		0.268		0.052		0.481
Faecalibacterium prausnitzii		0.044		-0.033		0.111		0.091		0.086		0.022
SCFA[e] (mM)												
acetate	0.397	0.715**	0.351	0.404	0.380	0.137	0.442	1.021**	0.294	0.790**	0.223	0.367
propionate		-0.705**		-0.613*		-0.099		-0.587*		-0.839**		-0.756*
isobutyrate		0.421		-0.004		0.721		0.768		0.853		0.096
butyrate		0.215		0.186		0.454		-0.042		0.008		0.248
isovalerate		-0.040		0.536		-1.239*		-0.520		-0.552		0.241

[a]Results based on lineal regression analysis. [b]R^2, coefficient of multiple determinations. [c]β, standardized regression coefficient. *$p \leq 0.05$ and **$p \leq 0.01$. [d]microbiota included: *Akkermansia, Bacteroides-Prevotella-Porphyromonas, Bifidobacterium, Clostridium* cluster XIVa, *Lactobacillus* group and *Faecalibacterium prausnitzii* as factors and age, energy intake, body mass index and physical activity as covariates or alternatively [e]SCFA: acetate, propionate, isobutyrate, butyrate and isovalerate as factors and age, energy intake, body mass index and physical activity as covariates.

Table 4. Results from the Lineal Regression Analyses in order to Estimate the Relationship between Blood Biomarkers and the Fecal Excretion of Total and those Fecal Phenolic Metabolites whose Concentration Differed Significantly among Tertiles[a].

	total phenolic content (μg/mL)		phenylacetic acid (μg/mL)		3-(3′-hydroxyphenyl) propionic acid (μg/mL)		phenylpropionic acid (μg/mL)		protocatechuic acid (μg/mL)		phthalic acid (μg/mL)	
	R^2 [b]	β[c]	R^2	β	R^2	β	R^2	β	R^2	β	R^2	β
TAC[d] (mM)	0.104	0.143	0.124	0.119	0.157	0.026	0.048	0.140	0.091	-0.070	0.098	-0.044
MDA[e] (μM)	0.128	0.264	0.194	0.370*	0.156	0.009	0.031	-0.030	0.097	-0.132	0.116	0.180
CRP[f] (pg/mL)	0.128	0.222	0.180	0.283*	0.157	-0.042	0.032	0.051	0.117	0.190	0.130	0.196
total cholesterol (mg/dL)	0.059	0.077	0.074	0.021	0.167	0.011	0.033	0.168	0.112	0.167	0.112	-0.127
HDL-cholesterol[g] (mg/dL)	0.057	-0.054	0.079	-0.079	0.167	-0.009	0.010	-0.027	0.092	0.058	0.105	-0.085
LDL-cholesterol[h] (mg/dL)	0.058	0.060	0.074	0.022	0.167	-0.027	0.029	0.150	0.124	0.199	0.105	-0.080
triglycerides (mg/dL)	0.076	0.158	0.083	0.100	0.186	0.150	0.035	0.171	0.098	-0.103	0.110	-0.111
serum glucose (mg/dL)	0.068	0.123	0.085	0.114	0.171	0.070	0.013	0.066	0.090	0.028	0.120	-0.156
TGF-β[i] (pg/mL)	0.231	0.398**	0.294	0.446**	0.162	0.081	0.044	0.123	0.164	0.291*	0.209	0.351**
IL-10[j] (pg/mL)	0.133	0.223	0.165	0.237*	0.156	-0.005	0.039	0.096	0.096	0.101	0.097	-0.031
IL-17[j] (pg/mL)	0.142	0.244*	0.173	0.254*	0.156	-0.005	0.041	0.105	0.100	0.119	0.097	-0.026
IL-8[j] (pg/mL)	0.143	0.353*	0.179	0.385*	0.165	-0.138	0.059	0.252	0.090	0.088	0.179	0.422*
TNF-α[k] (pg/mL)	0.088	-0.060	0.111	-0.003	0.156	0.002	0.054	-0.173	0.086	-0.005	0.100	-0.070
IL-12[j] (pg/mL)	0.113	-0.185	0.126	-0.135	0.156	0.008	0.075	-0.236	0.094	-0.099	0.106	-0.111

[a]Results based on lineal regression analysis adjusted by age, energy intake, body mass index and physical activity. [b]R^2, coefficient of multiple determinations. [c]β, standardized regression coefficient. *$p \leq 0.05$ and **$p \leq 0.01$. [d]TAC, total antioxidant capacity. [e]MDA, malondialdehyde. [f]CRP, C-reactive protein. [g]HDL, high-density lipoprotein. [h]LDL, low-density lipoprotein. [i]TGF, transforming growth factor. [j]IL, interleukin. [k]TNF, tumor necrosis factor.

FIGURE GRAPHICS

Figure 1

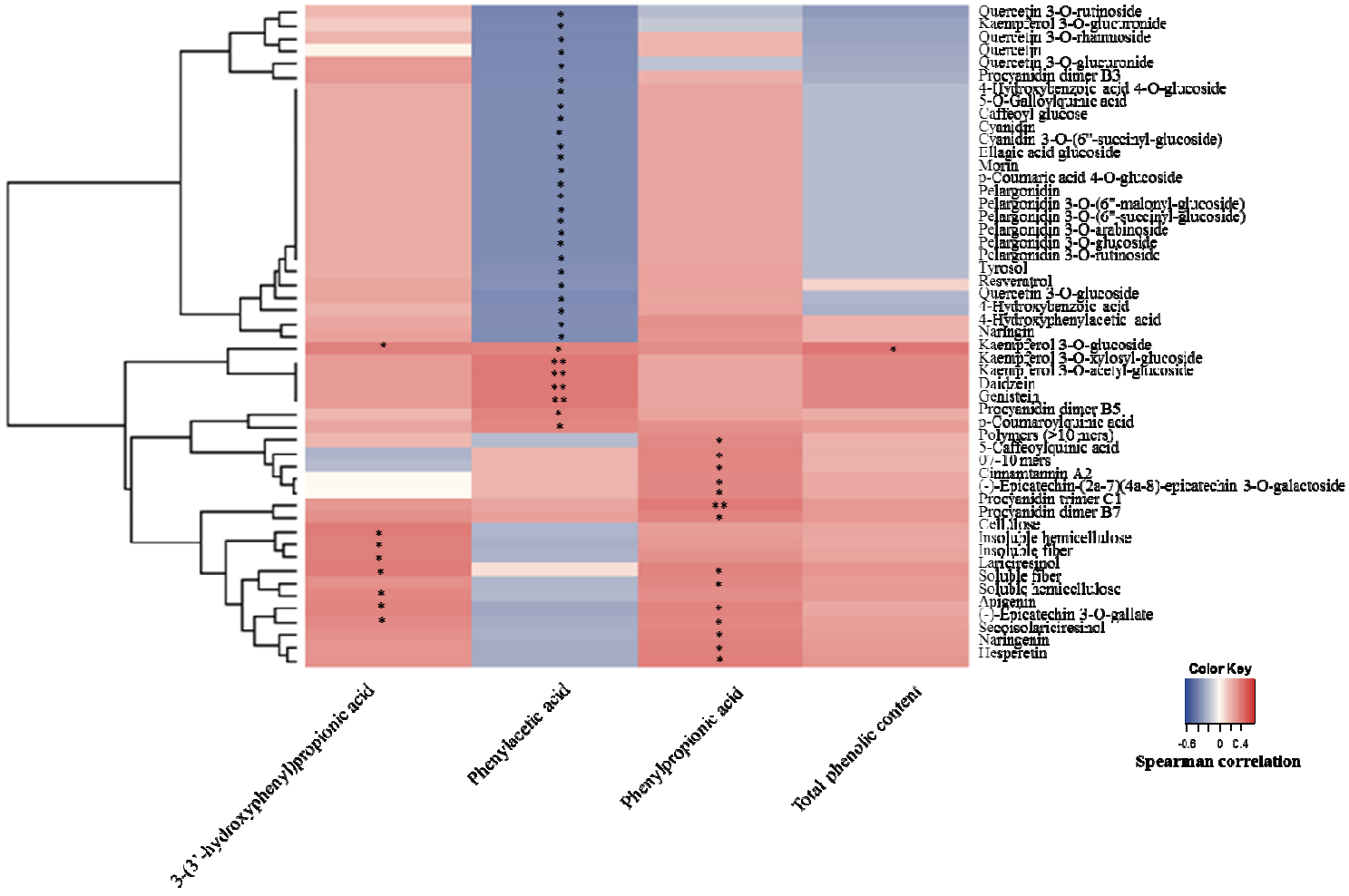
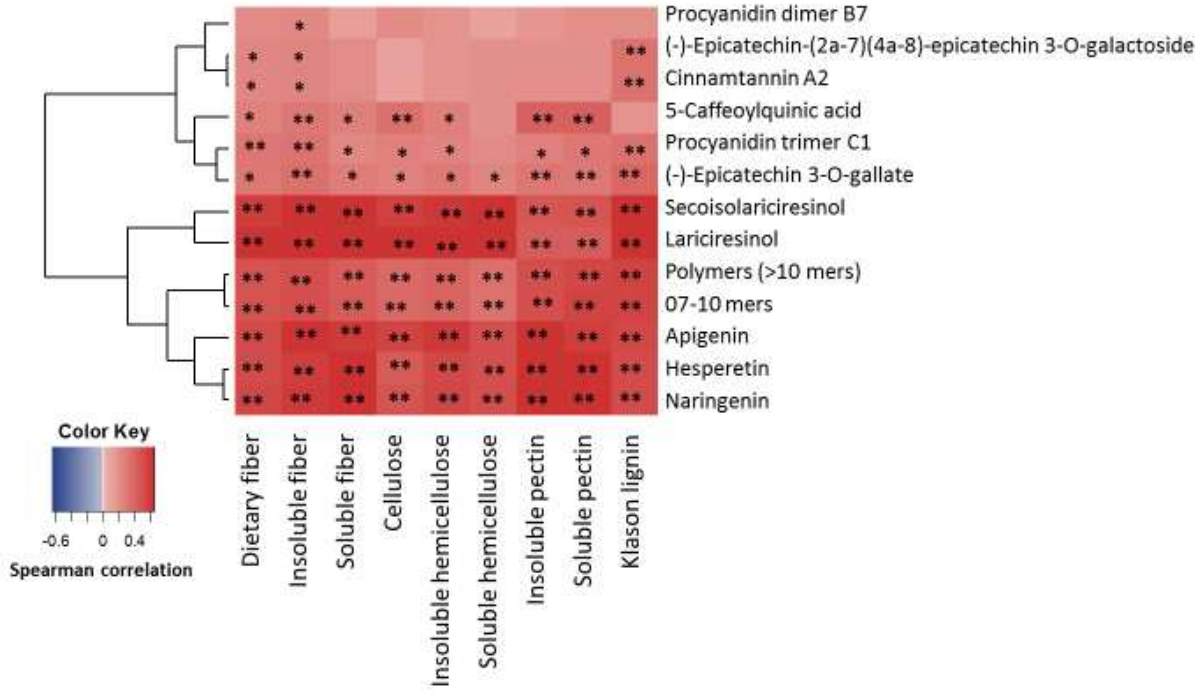


Figure 2


Procyanidin dimer B7
(-)-Epicatechin-(2a-7)(4a-8)-epicatechin 3-O-galactoside
Cinnamtannin A2
5-Caffeoylquinic acid
Procyanidin trimer C1
(-)-Epicatechin 3-O-gallate
Secoisolariciresinol
Lariciresinol
Polymers (>10 mers)
07-10 mers
Apigenin
Hesperetin
Naringenin
Dietary fiber
Insoluble fiber
Soluble fiber
Cellulose
Insoluble hemicellulose
Soluble hemicellulose
Insoluble pectin
Soluble pectin
Klason lignin
Color Key
-0.6 0 0.4
Spearman correlation

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